SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Fly Stocks and Tissue Preparation

Flies (*Drosophila melanogaster*) used in this study were doubly mutant for *yellow* and *white* and had been cultured in the laboratory of Matthew Scott for several years. Approximately fifty 0 to 8 hour-old first instar larvae were transferred to standard molasses medium in standard culturing vials and were placed at 25° C. This density of larvae enabled unimpeded developmental timing, and wandering third instar male larvae were selected for salivary gland dissection. Larvae were rinsed in ~20 mL of room temperature PBS and then transferred to room temperature PBS in a dissection dish. Larvae were inverted over the tip of a pair of forceps to expose the salivary glands as previously described (Hafer and Schedl, 2006). Carcasses with exposed salivary glands were then rinsed in ~100 mL of room temperature PBS to remove residual food that may have been in the gut. Twelve to fifteen larval carcasses were placed into 1.5 mL of freshly made fixative (2% EM grade paraformaldehyde diluted into PBS from a newly opened 20% ampule (Electron Microscopy Sciences)) in 2 mL microcentrifuge tubes. Carcasses were fixed for 10 minutes on a nutator at room temperature. Fixation was quenched by adding freshly made glycine from a 2.5 M stock to a final concentration of 150 mM followed by mixing on a nutator for 5 minutes at room temperature. The fixative/glycine mix was removed and carcasses were rinsed 3 times with 1 mL of PBS. Carcasses were kept on ice until a total of 25-30 carcasses had been fixed. Then, the bulk of the fat body tissue was dissociated from the salivary glands, and the glands were finely dissected away from carcasses in room temperature PBS and placed into ice cold PBS in a siliconized microcentrifuge tube. Once 50 glands had been collected in ice cold PBS, the PBS was removed from the gland carrying tube, and glands were snap frozen in liquid nitrogen and stored at -80° C.

Hi-C

Hi-C was performed using a tethering approach thereby increasing the signal-to-noise ratio as previously described (Kalhor et al., 2012). This method generates identical results as that of conventional, dilution Hi-C (Kalhor et al., 2012). Fifty or one hundred salivary glands

(in aliquots of 50 glands), thawed on ice, were resuspended in 100 µL per aliquot of lysis buffer (50 mM Bis-Tris-HCl pH 6.0, 100 mM NaCl, 10 mM $MgCl_2$, 0.1% SDS) plus protease inhibitors (1 mM PMSF, 2 mM benzamidine, 2 µM pepstatin A, 0.6 µM leupeptin) and incubated on ice for 15 minutes. The cell mixture was transferred to a siliconized 1 mL Dounce homogenizer, lysed with 15 strokes of pestle B (loose-fitting pestle), and placed on ice for 1 minute. Then another 15 strokes with pestle B were applied. Lysis was performed in a 4° C room. The sample was transferred to a 1.5 mL siliconized microcentrifuge tube and the homogenizer rinsed twice with 130 µL of lysis buffer without protease inhibitors and these washes were combined with the lysate. The sample was incubated at 65° C for exactly 10 minutes to solubilize the cross-linked chromatin and then immediately cooled on ice. 140 µL of 25 mM EZ-link Iodoacetyl-PEG2-Biotin (IPB; in lysis buffer without protease inhibitors; Thermo Scientific) was added to each tube and then incubated at room temperature protected from light while mixing for 1 hour to biotinylate cysteine residues. 60 µL of 10% Triton X-100 was added to each tube and mixed by gently inverting the tube. 16 µL of DpnII (50 U/µL, New England Biolabs) was added to each tube and then incubated overnight at 37° C while mixing. 129.8 µL of 10% SDS was added to each sample and then incubated at 65° C for exactly 30 minutes to inactivate the restriction enzyme and then immediately cooled on ice. All samples were pooled, added to a 20 kD MWCO, 0.5-3 mL Slide-A-Lyzer Dialysis Cassette (Thermo Scientific), and dialyzed at room temperature against 4 L dialysis buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA). After 2 hours the dialysis buffer was replaced with fresh buffer and dialysis continued for another 2 hours. The dialyzed sample was transferred to a 1.5 mL siliconized microcentrifuge tube and an aliquot was frozen at -20° C as a digestion-only control. The sample was then divided into 5 equal volume aliquots in 1.5 mL siliconized microcentrifuge tubes. 400 µL of Dynabeads MyOne Streptavidin T1 beads (Life Technologies) were washed with 2 mL of PBS + 0.01% Tween 20 (PBST) and a 400 µL aliquot of the bead suspension in PBST was added to each sample tube. The samples were incubated at room temperature while mixing for 30 minutes. Neutralized IPB (i.e. treated with β-mercaptoethanol) was then added to each tube and incubated at room temperature while mixing for 15 minutes to fully titrate all of the biotin binding sites. The beads were washed with 600 µL of PBST, 600 µL of wash buffer 1 (10 mM Tris-HCl pH 8.0, 50 mM NaCl, 0.4% Triton X-100) and then resuspended in 100 µL of wash buffer 1 per tube. Tube 1 was labeled as a 3C control sample and tubes 2-5 as Hi-C samples. 85 µL of water, 1 µL of 1 M $MgCl_2$, 10 µL of 10X NEBuffer 2 (New England

Biolabs), and 4 µL of 10% Triton X-100 was added to the 3C control sample. 95 µL of a fill-in master mix (267 µL water, 4.5 µL 1 M $MgCl_2$, 45 µL 10X NEBuffer 2, 3.15 µL 10 mM dTTP, 3.15 µL 10 mM dGTP, 3.15 µL 10 mM dCTP, 78.8 µL 0.4 mM Biotin-14-dATP (Life Technologies), 18 µL 10% Triton X-100) was added to each Hi-C tube and mixed by gently inverting each tube. 25 U of DNA Polymerase I, Large (Klenow) Fragment was added to each Hi-C tube and then all tubes were incubated at 37° C while mixing for 2 hours. Reactions were quenched with EDTA and the beads washed with wash buffer 2 (50 mM Tris-HCl pH 7.4, 0.1 mM EDTA, 0.4% Triton X-100) per tube. 4.498 mL of a ligation mix (18.989 mL water, 2.588 mL 10% Triton X-100, 2.588 mL 500 mM Tris-HCl pH 7.5 + 100 mM $MgCl_2$, 278 µL 10 mg/mL BSA (New England Biolabs), 278 µL 100 mM ATP (Sigma; freshly prepared), 278 µL 1 M DTT (US Biological; freshly prepared), 1 µL 0.111 ng/µL SmaI digested pUC19 (to serve as a ligation control)) was added to each tube and mixed by gently inverting each tube. 4,000 cohesive end U of T4 DNA ligase (New England Biolabs) was added to each Hi-C sample, 800 cohesive end U was added to the 3C sample, and then all tubes were incubated at 16° C while mixing overnight. Reactions were quenched with EDTA and the beads resuspended in 300 µL extraction buffer (50 mM Tris-HCl pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.2% SDS). 500 µg of proteinase K (New England Biolabs) was added to each tube (including the digest only control) and then incubated at 65° C. After three hours an additional 500 µg proteinase K was added to each tube and the digestion continued overnight at 65° C. The beads were collected against the tube wall, the supernatant recovered by aspiration and transferred to fresh 1.5 mL screw-top siliconized microcentrifuge tube per sample. DNA was purified by two extractions with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1 v/v/v) followed by a single extraction with chloroform. 1/10 volume 3M sodium acetate pH 5.2, 50 µg glycogen, and 2.5 volumes 100% ethanol were added to each sample and incubated at -80° C for one hour. Samples were pelleted by centrifugation and each pellet was washed with 1 mL of 70% ethanol (stored at -20° C), pelleted again by centrifugation, air dried at room temperature, and, before being completely dried out, resuspended in TE buffer (1X: 10 mM Tris pH 8.0, 1 mM EDTA) per pellet and digested with RNase A. All subsequent steps were then performed on the Hi-C sample. Unligated biotinylated ends were removed from the Hi-C sample by treating the DNA with 30 U T4 DNA Polymerase (New England Biolabs) in the presence of 25 µM dGTP at room temperature for four hours. The reaction was quenched with EDTA and then the DNA sheared in a Covaris S2 instrument (Covaris) at a duty cycle of 10%,

intensity of 5, cycles/burst of 200 for a total of 180 seconds. DNA was ethanol precipitated and resuspended in a volume of 55.5 µL 0.1X TE buffer. DNA was end-repaired and adaptor-ligated by following "NEBNext End Prep" and "Adaptor Ligation" in the NEBNext Ultra DNA Library Prep Kit for Illumina (New England Biolabs). 4 µL of Dynabeads MyOne Streptavidin C1 beads (Life Technologies) was added to 46 µL of 1X B&W buffer + 0.05% Tween 20 (2X Bind and Wash (B&W) buffer: 10 mM Tris-HCl pH 7.5, 2 M NaCl, 1 mM EDTA) and then washed twice with 100 µL of 1X B&W buffer + 0.05% Tween 20 before resuspending in 86.5 µL of 2x B&W buffer. The adaptor-ligated sample was added to the bead suspension and then incubated at room temperature while mixing for 30 minutes. The beads were washed with twice with 100 µL of 1X B&W buffer + 0.05% Tween 20, twice with 100 µL of TE buffer, and then resuspended in 8 µL of 95% freshly deionized formamide + 10 mM EDTA. DNA was eluted from the beads by heating to 90° C for 10 minutes and the eluted library was collected from the beads using a magnet. We found that this step increased PCR yield and library diversity. The number of PCR cycles was then titrated for efficient amplification of the Hi-C library. Four PCR reactions, each containing 6 µL of water, 1 µL of eluted Hi-C library, 1 µL of 10 mM $MgCl_2$, 1 µL of 2.5 µM NEBNext Universal PCR Primer (New England Biolabs), 1 µL of 2.5 µM NEBNext Index Primer (New England Biolabs), and 10 µL of NEBNext High-Fidelity 2X PCR Master Mix (New England Biolabs) were thermocycled at 98° C for 30 seconds, followed by 9, 12, 15, or 18 cycles of 98° C for 10 seconds, 65° C for 30 seconds, 72° C for 30 seconds, followed by a final extension at 72° C for 5 minutes, and then held at 4° C. The PCR reactions were then run on a 1X TBE 2% agarose gel, stained with SYBR Gold (Life Technologies), and the optimal number of PCR cycles determined by observing linear amplification of the adaptor-ligated Hi-C library (size range of 200-400 bp). Four identical PCR reactions, using the conditions and the number of PCR cycles determined above, were performed on the remaining bead-eluted Hi-C library. The PCR product was then purified following "Cleanup of PCR Amplification" in the NEBNext Ultra DNA Library Prep Kit for Illumina (New England Biolabs) except 0.7 volumes of AMPure XP beads were used. DNA concentration was determined using a Qubit dsDNA HS Assay Kit (Life Technologies) and DNA integrity determined by running an aliquot on a Bioanalyzer High Sensitivity DNA Chip (Agilent). The library was accurately quantified using a KAPA Library Quantification Kit (KAPA) and then subjected to paired-end sequencing on an Illumina MiSeq or NextSeq 500 instrument.

Characterization of Polytene Hi-C Procedure

Tissues were prepared as described above, but were cross-linked with 0, 0.25, 0.5, 1 or 2% paraformaldehyde. The samples were then processed for Hi-C up to and including the DpnII digestion step. Cross-links were then reversed and the DNA purified as described above. qPCR was then performed with primers flanking four DpnII restriction sites at the 72D locus. The amount of product was normalized to a primer pair at the same locus not flanking a restriction site. From these results (Figure S1A) we determined that 2% paraformaldehyde provides a satisfactory amount of cross-linking, yet still allows for a substantial portion of restriction sites to be cut and therefore available for subsequent ligation.

To ensure efficient biotin incorporation a PCR-digest assay was performed similarly to that previously described (Belton et al., 2012). Upon fill-in of a DpnII site and subsequent blunt-end ligation a ClaI site is formed. It is important to note that fill-in of a DpnII site also retains the DpnII site and therefore DpnII is expected to cut both 3C and Hi-C templates. MboI is an isochizomer of DpnII and is compatible with same buffer used for restriction digestion with ClaI. The efficiency of biotin incorporation is calculated as described in Figure S1B.

To ensure ligation under the less favorable Hi-C conditions (compared to a routine ligation reaction used for cloning, for instance) a small amount of SmaI digested, unbiotinylated pUC19 plasmid was spiked into the samples during the ligation step. After purifying the DNA, qPCR was performed with primers flanking the cut site (black and blue primers in Figure S1C) and this amount of product was compared to qPCR with primers that do not flank the cut site (black and red primers in Figure S1C). The ratio of the amount of products from these two reactions was normalized to the amount of cut plasmid (determined by the above described qPCR method on cut, unligated plasmid). Although ligation efficiency may be different for the cross-linked, biotinylated Hi-C template, this assay assures that the ligase is active under the Hi-C reaction conditions.

To ensure biotinylated, unligated ends (aka “dangling ends”) were removed from the Hi-C templates a “dangling ends” assay was performed similarly to that previously described (Belton et al., 2012) but ClaI was used as the restriction enzyme to assess the proportion of dangling ends. The amount of dangling ends present is approximately inversely related to the amount of library cut by ClaI (Figure S1D).

Hi-C Analysis

Sequencing reads were mapped to chromosomes X, 2L, 2R, 3L, 3R, 4, and M of the *Drosophila melanogaster* genome (BDGP Release 5; downloaded from UCSC Genome Browser (http://genome.ucsc.edu/index.html) on 22 August 2013). Next reads were assigned to DpnII restriction fragments. Reads mapping to the same restriction fragment that represent either self-ligation products (self-circles), non-ligation products (dangling ends) or ligation products between paired and aligned homologs/chromatids were removed as they do not represent informative chromatin interactions. Reads separated by less than the maximum molecule length in the Hi-C library are also removed as they may represent spuriously pulled down products (Imakaev et al., 2012). Reads that are retained after these steps represent valid pairs. Pairs with one read within 4 bp (5 bp for libraries digested with HindIII) of a restriction site, duplicate read pairs, restriction fragments with extraordinarily high counts (highest 0.5% of fragments), or very large (> 100 kb; potentially repetitive or poorly assembled sequences) or small (<100 bp; comparable to the read length) restriction fragments were also removed as previously described (Imakaev et al., 2012).

The genome was binned into 15 kb bins, unless otherwise indicated, and filtered fragments were assigned to each of the 15 kb bins based on the midpoint of the restriction fragment. At the bin-level, bins where less than half of a bin was sequenced (very low coverage bins), the lowest 1% of bins, and the highest 0.05% of interchromosomal contacts were removed to avoid vastly underrepresented or overrepresents regions due to, for example, PCR artifacts as previously described (Imakaev et al., 2012). Biases due to restriction fragment

density, GC content, mappability, and copy number were corrected for using an iterative approach as previously described (Imakaev et al., 2012). This approach ensures that the coverage across the genome is uniform (Imakaev et al., 2012). Finally, heatmaps were normalized so that each row and column summed to 1. Results from three biological replicates were highly correlated at bin sizes of 15 kb or larger and therefore datasets were merged after filtering. All of the above steps were performed using the *hiclib* python library from https://bitbucket.org/mirnylab/hiclib. All subsequent calculations were performed on these normalized heatmaps, except for the determination of background reads as this calculation relies on read counts not contact probabilities (Dixon et al., 2012). Mathematica 9 (Wolfram) or the *matplotlib* python library were used to display all heatmaps. Here we refer to distant or long-range interactions as loci separated by 1 Mb or more.

The number of background intermolecular reads was determined as previously described (Dixon et al., 2012). In brief, interactions are not expected between the mitochondrial and nuclear genome so any read pairs between these genomes represent random, background intermolecular ligation events. Therefore, the number of mitochondrial to nuclear genome reads, corrected for the mitochondrial-nuclear interaction space (in kb^2), and the ratio of mitochondrial to nuclear genome copy number (here determined empirically by qPCR) is used to estimate the number of background intermolecular reads (Dixon et al., 2012).

The correlation between datasets was determined as previously described (Dixon et al., 2012). In brief, matrices were flattened to vectors and the correlation between the two vectors was calculated, only considering interactions at most 50 bins apart to assess agreement between datasets at the length scale of TADs and not at the length scale of compartments since compartments are not necessarily conserved between datasets. Since the Hi-C procedure applied to *Drosophila* embryos did not enrich for ligation products (Sexton et al., 2012) we removed bins along and touching the diagonal when calculating correlations involving embryonic Hi-C data.

The directionality index (DI) was determined as previously described (Dixon et al., 2012) using software from http://yuelab.org/hi-c/download.html:

$$\mathrm{DI} = \left(\frac{B-A}{|B-A|}\right)\left(\frac{(A-E)^2}{E} + \frac{(B-E)^2}{E}\right)$$

where *A* is the number of normalized contacts from a given 15 kb bin to the upstream 750 kb, *B* is the number of normalized contacts from a given 15 kb bin to the downstream 750 kb, and *E* is the number of expected normalized contacts under the null hypothesis, $E = \frac{(A+B)}{2}$. In brief, DI measures the directional bias for each Hi-C bin using a modified chi-squared statistic (Dixon et al., 2012). DI is computed genome-wide and is displayed for chromosomal features of interest.

Matrix transformations to detect compartments were done as previously described (Lieberman-Aiden et al., 2009). In brief, an expected heatmap is computed for each chromosome by determining the total number of observed interactions separated by a distance, *d*, divided by the total number of loci separated by *d* for all possible *d* (Figure S3A). For each iteration, *d* is increased by a factor of 1.05 to account for very few interactions being observed at very large *d*, thereby ensuring that when *d* is large the low density of interactions does not affect the calculation. Dividing the observed heatmap by the expected heatmap generates the observed/expected heatmap. The observed/expected heatmap is sharpened by realizing that if two loci are in close spatial proximity, that is in the same compartment, and their linear neighbors are also in the same compartment then they should share the same interaction preferences. Therefore, the interaction profile of neighboring linear loci should be correlated if they are in the same compartment. The Pearson correlation coefficient, *r*, between the i^{th} row and j^{th} column of the observed/expected heatmap is calculated to generate the Pearson correlation heatmap. Compartment analysis was performed using 100 kb bins (resolution) such that more interactions fall within one bin, thereby generating reliable contact heatmaps for distant interactions.

To identify TADs at 15 kb resolution we noticed that the signal along the diagonal within TADs was much less than in regions between TADs and therefore could be used as a means to identify TADs. For each chromosome the value midway between the mean of the values in the lowest two deciles of the contact probability along the diagonal and the mean of the values in the highest two deciles of the contact probability along the diagonal was used as a threshold for a low-pass filter to identify TADs. Given the 15 kb resolution of the Hi-C data, TADs were further required to have a minimum size of 75 kb (i.e. 5 bins in the Hi-C heatmap) to satisfy the Nyquist sampling criterion and to account for noise. Regions of chromosomes not associated with TADs are identified as "between TADs".

Reliable DNA sequence coordinates of bands identified by FISH and non-histone protein localization were taken from (Belyaeva et al., 2012; Vatolina et al., 2011), but only bands equal to or larger than 75 kb (i.e. 5 bins in the Hi-C heatmap) were used, given the 15 kb resolution of the Hi-C data. This criterion discarded seven bands out of a total of 68. DNA coordinates of cytological locations corresponding to puff loci were taken from http://flybase.org/. Highlighted puffs are present throughout the state of development when we collected the larvae, i.e. the wandering third instar stage. This corresponds to puff stages 5-8 (Ashburner et al., 2011). We only considered puffs present throughout all of these stages, that score + (range from – to ++ which denotes size of the puff, ++ being the largest) throughout stages 5-8, and ++ in at least two of the four puff stages during this time of development (Ashburner et al., 2011). This restricts the analysis to the largest, most prominent puffs to ensure than any effect would be manifest across the cell population and therefore apparent in the Hi-C heatmaps.

deepTools v1.5.8.1 (Ramírez et al., 2014) was used to generate heatmaps comparing polytene TAD bodies with polytene band, Kc167 cells TAD, or embryonic nuclei TAD bodies. Hi-C data and TADs from Kc167 cells (Hou et al., 2012) (40.7 million contacts) and embryonic nuclei (Sexton et al., 2012) (24.1 million contacts) were previously published and obtained from NCBI's Gene Expression Omnibus using accession numbers GSE38468 and GSE34453, respectively.

To make statistical comparisons between chromosomal features (e.g. polytene TADs and bands or polytene and diploid TADs) we match features in A (e.g. polytene TADs) with those in B (e.g. bands). We use two different measures of the match: (1) the overlap of all the features in A with all those in B and (2) the total length of unmatched regions in A and B (in 100 kb). This is done numerically by converting each list of chromosomal features to a bit string and simply counting. Figure S4A shows a simple example of two lists along a model chromosome converted to bit strings. The AND operator gives "1" only if both A and B are "1"; the OR operator gives "1" when either A or B is "1". The total overlap score is normalized by dividing by the smaller of the total length of the features in lists A and B, which limits its maximum value to 1.0. We then use bootstrapping to estimate the distribution of the two scores and so get the significance of the actual match of A and B. This is done by shuffling the order of the features in list B while ensuring that features remain on their respective chromosome and are not merged in the shuffle, and then converting the shuffled chromosome B to a bit string (Figure S4A) to allow its match scores to be computed. The normalized overlap and total length of unmatched regions between list A and randomized list B are then calculated. Note that our shuffling is conservative in that it is performed per chromosome such that for each chromosome the number of features and the size distribution of features in list B is preserved.

This procedure is repeated 10,000 times generating a new set of randomized features in list B each time, thereby generating a distribution of random scores. The Z-score and *p*-value for each score is then determined by comparing the observed match of list A with list B to that of the distribution of shuffled scores. If the observed match is better than random, the Z-score for the normalized overlap should be positive (more overlap than random), whereas the Z-score for the unmatched regions should be negative (fewer unmatched regions than random). The random distribution is normally distributed as evidenced by the R^2 value between the random distribution and a Gaussian distribution defined by the mean and standard deviation of the random distribution being > 0.99 in every case. The Z-scores and *p*-values reported in the main text reflect holding polytene TADs constant (i.e. list A) and randomizing the compared chromosomal feature (i.e. list B). In all cases swapping list A for list B and repeating the above analysis yielded nearly identical results, indicating that the analysis is robust in regards to which list is randomized (Figure S4B). Furthermore, when

comparing two lists, the absolute value of the Z-scores and the *p*-values for the normalized overlap score and the aggregate distance between boundaries score were very similar (Figure S4B) indicating that the statistical significance of the results are robust regardless of which score is used.

In addition to quantitatively comparing the boundaries between chromosomal features (e.g. polytene TADs and bands or polytene and diploid TADs), we sought to also to quantitatively compare if entire domains are conserved between two sets. This is important because conservation of domain boundaries does not necessarily indicate conservation of entire domains because, for instance, if a domain in set A is split in half in set B the boundaries in set A will overlap with those in set B even though domain has been split in half (Rao et al., 2014). In addition, when comparing boundaries or entire domains a seemingly arbitrary cut-off is often used to determine if boundaries or domains are conserved (Dixon et al., 2012; Hou et al., 2012; Rao et al., 2014). We therefore sought to develop an unbiased estimate of how well entire domains are conserved.

To this end, we consider two domains, one in dataset A and another in dataset B (Figure S6A). The location of each domain can be precisely represented as a vector, *v*, from the origin to the corner of each domain (since the domain is symmetric either the upper-left or lower-right corner can be chosen, but the same corner must be used in both datasets). We then define the corner vector, v_{Corner}, as the vector from the corner of a domain in A to the corner of a domain in B. We define the Euclidean distance between the two domains as the corner distance, which is the magnitude of the corner vector (Figure S6A):

$$\|v_{Corner}\| = \|v_A - v_B\|$$

If entire domains are conserved between dataset A and dataset B then the corner distance should be minimal.

To compare datasets A and B we computed the corner distance between a domain in A with the closest (smallest corner distance) domain in B for all A domains. We then use bootstrapping to estimate the distribution of corner distances and so get the significance of

the conservation of entire domains between datasets of A and B. This is done by shuffling the domains in list B, as described above, and then computing the corner distance between a domain in A with the closest shuffled domain in B for all A domains. This is repeated 10,000 times, as above. We then compute the cumulative distribution for both the observed corner distances and the mean of the shuffled corner distances and use the Kolmogorov-Smirnov test to determine if the two distributions significantly differ (Figures S5B-D).

FISH

Polytene chromosome spreads were prepared as previously described (Kennison, 2000). FISH probes were synthesized by PCR using primers listed below, digested overnight with AluI and MboI, and end-labeled with aminoallyl dUTP (ARES DNA Labeling Kit, Life Technologies) using terminal deoxynucleotidyl transferase as previously described (Dernburg, 2000). End-labeled fragments were conjugated to either Alexa Fluor 488 (TAD border probes) or Alexa Fluora 555 (TAD body probes) using an ARES DNA Labeling Kit (Life Technologies) following the manufacturer's instructions. FISH on polytene chromosomes was performed as previously described (Pardue, 2000), except the hybridization buffer was 2X SSC, 10% (w/v) dextran sulfate, 50% formamide, 0.8 mg/mL salmon sperm DNA and hybridization was carried out overnight at 37° C. After washing away unhybridized probe, slides were counterstained with DAPI. Slides were imaged on a Zeiss Axio Imager.M1 microscope using a Zeiss Plan-Apochromat 63X/1.4 NA oil DIC objective and images acquired with AxioVision 4.8.2.0 software at an image bit depth of 16 bits. Images were collected on a Zeiss AxioCam HRm Rev3 camera with a 54.2 nm pixel size. Appropriate filters for fluorochromes Alexa Fluor 488, Alexa Fluor 555, and DAPI were used. The DAPI and FISH channels were merged, the DAPI channel pseudocolored blue, the TAD border FISH channel pseudocolored green, the TAD center FISH channel pseudocolored red each using a linear LUT that covers the full range of data, and the images converted to TIFF format using ImageJ.

Primer Sequences for Synthesizing FISH Probes

Chromosome 2L

Centromere Proximal TAD Border Probe:

5' – TGTTGTGTTTCGCCCTCACT – 3' and 5' – ACAGAATATCCGTGGCCGAC – 3'

TAD Center Probe:
5’ – CTGGCTGTAGGAGGGGTTTG – 3’ and 5’ – ACAACTTCCACGTCGAGTCC – 3’
Centromere Distal TAD Border Probe:
5’ – GGAGGAGTCCAAAAAGGCGA – 3’ and 5’ – AGCATGCCGACGATCGTATT – 3’

Chromosome 3L

Centromere Proximal TAD Border Probe:
5’ – CGCACTCCATTCTCCACCTT – 3’ and 5’ – GTGTGTCGCACTCTCCTGAT – 3’
TAD Center Probe:
5’ – ATTTCAAGCGAGTGGGGAGG – 3’ and 5’ – CATTTGACCCCAGAGTGGCT – 3’
Centromere Distal TAD Border Probe:
5’ – ATCTGGCTATCTGCGCAGAC – 3’ and 5’ – AAGTGCCGAGTTTGAGGAGG – 3’

Chromosome 3R

Centromere Proximal TAD Border Probe:
5’ – TGATGGGGTTTTCGATGCCA – 3’ and 5’ – AGAAGTGTGCCATTCGAGGG – 3’
TAD Center Probe:
5’ – CGGGCCGCAGTTTGTTTTTA – 3’ and 5’ – CCCGCATCGCACTCTCTATT – 3’
Centromere Distal TAD Border Probe:
5’ – TGCAGTGAGAAGACCACGTC – 3’ and 5’ – ATGTGTTGGGTGGCGATCAT – 3’

Copy Number and Influence on Hi-C

Since select regions of polytene chromosomes can be underreplicated with respect to the rest of the chromosome we undertook two approaches to determine any affect this would have on the Hi-C results.

First, underreplication does not occur on the X chromosome of males due to dosage compensation so the copy number is uniform across the X chromosome in male larvae (Alekseyenko et al., 2002). We only performed Hi-C on male larvae and the pattern of contacts observed by Hi-C on the X chromosome is similar to that on the autosomes, so underreplication does not affect the Hi-C signal.

Second, we experimentally determined the underreplication state by purifying total genomic DNA from unfixed, male salivary gland tissue manually dissected as for Hi-C and from 0-6

hour embryos. In the first six hours of embryogenesis, endoreplication does not occur and all cells are diploid (Smith and Orr-Weaver, 1991). We prepared Illumina libraries from both samples using a NEBNext DNA Library Prep Reagent Set for Illumina (New England Biolabs) and subjected them to 50 bp single-read sequencing on an Illumina HiSeq 2000 instrument. Reads were mapped to the *Drosophila melanogaster* reference genome (BDGP Release 5; downloaded from UCSC Genome Browser (http://genome.ucsc.edu/index.html) on 22 August 2013) using bowtie2 and then duplicate reads were removed using picard. deepTools v1.5.8.1 (Ramírez et al., 2014) was used to quantify the $\log_2$ ratio of the sequencing-depth normalized salivary gland read coverage to embryonic read coverage in 15 kb bins (same as the Hi-C bins) across the genome. The coverage across the salivary gland X chromosome was corrected by a factor 3/2 to account for all of the larvae being male and the mixed male:female population in the 0-6 hour embryos. The genome-wide, sequencing-depth normalized mean coverage for male salivary glands was 229 reads/bin and for 0-6 hour embryos 219 reads/bin. Comparing the coverage from salivary glands to that from 0-6 hour embryos controls for any unexpected biases during library preparation and sequencing. Although there are difference in copy number across certain regions of the genome, they are minimal at 15 kb resolution and do not appear to affect the Hi-C result (Figure S3).

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